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13. ABSTRACT ***(Maximum 200 Words)***

Cancer incidence rises exponentially with age. This project tests the hypothesis that cellular senescence of stromal fibroblasts contributes to the age-dependent increase in breast cancer by creating a more permissive environment for the expression of malignant phenotypes by breast epithelial cells.

Here, we report that both mouse and human immortal pre-malignant breast epithelial cell lines increase 2 to 5 times their proliferation in the presence of senescent, compared to presenescent, human fibroblasts. Both soluble and insoluble secreted factors contribute to senescent fibroblasts' effect on epithelial cell proliferation. The effect of senescent fibroblasts on breast epithelial cell proliferation is observable even when they are present as only 10% of a mixed population of presenescent and senescent fibroblasts. In addition, fibroblasts induced to undergo premature senescence by oncogenic ras or p14/ARF expression stimulate epithelial cell proliferation in a manner similar to that observed with replicatively senescent fibroblasts. Most importantly, when senescent fibroblasts are co-injected into nude mice together with nontumorigenic epithelial cell lines, they stimulate tumorigenesis resulting in a higher frequency of tumors and larger tumor size relative to controls co-injected with presenescent fibroblasts. Taken together, these results support our hypothesis that cellular senescence of stromal fibroblasts may contribute to breast tumorigenesis.

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Annual Report

Role of Senescence in Breast Cancer

Introduction

Age is the main risk factor for the majority of human cancers, including breast cancer. The goal of this project is to investigate whether cellular senescence contributes to this age-related rise in cancer frequency. Specifically, we want to test the hypothesis that cellular senescence of stromal fibroblasts alters the environment of breast epithelial cells such that it becomes permissive for the expression of malignant phenotypes. With this in mind, we created cell culture systems that allowed us to explore the influence of senescent fibroblasts on breast epithelial cell behavior. In particular, we developed direct and indirect co-culture systems and methods to quantify changes in breast epithelial cell proliferation under these conditions. In addition, we confirmed some of the results observed in our *in vitro* co-culture system by *in vivo* tumorigenicity studies in nude mice. Using this experimental system we hope to contribute to understanding the mechanism by which the aging creates a tissue environment that can contribute to breast cancer pathogenesis.

Body of the Annual Summary

Senescent fibroblasts stimulate proliferation of pre-malignant immortalized ("initiated") epithelial cells. Age is the main risk factor for the majority of human cancers. One way in which cellular senescence can potentially contribute to this age-related rise in cancer frequency is through alteration of epithelial cell microenvironment. Specifically, phenotypic changes in stromal fibroblasts induced by replicative senescence may give rise to a microenvironment permissive for the expression of malignant phenotypes.

To test this hypothesis, WI38 human fetal lung fibroblasts from presenescent and senescent cultures were grown 1-3 days, and epithelial cells were dispersed on top of the fibroblasts and grown in direct co-culture for 5-8 days in serum-free medium. Co-cultures were evaluated for the size of the colonies after rhodanile staining, or stained with DAPI and used for quantification of the epithelial cell number by epifluorescence and an image analysis program (developed in collaboration with Dr. Stephen Lockett, head of the Bioimaging and Microscopy laboratory in the Life Sciences Division of LBNL). Five fields of the co-culture plate were analyzed for each sample. Results obtained with DAPI staining were confirmed with analysis of green epifluorescence, resulting from GFP expression in HaCAT and SCp2 cells.

To ensure that the effects observed are not exclusive to a one cell line, experiments were performed in parallel using three different immortalized cell lines which retained ability to differentiate: human and mouse mammary epithelial cells (S1 and SCp2), and human keratinocytes (HaCAT). All three epithelial cell types examined form larger colonies on the senescent compared to presenescent fibroblasts. The increase in colony size was most prominent for SCp2 cells, amounting to 3 to 6 fold higher number of epithelial cells on senescent relative to presenescent fibroblasts. HaCAT and S1 cells consistently showed 2 to 3 fold increases in cell number when grown on senescent relative to presenescent fibroblasts. This difference in growth on presenescent and senescent fibroblasts typically became obvious 3-5 days after plating, reaching a maximum on day 7 or 8 of co-culture. Under conditions used for co-culture,

control cells plated without fibroblasts showed little (HaCAT) or no growth (SCp2). Taken together, these findings suggest that senescent fibroblasts stimulate proliferation of immortalized epithelial cells of various origins and thus may contribute to epithelial malignant transformation in variety of mitotic tissues.

Presence of only 10% senescent fibroblasts stimulates SCp2 proliferation. Although it has been shown that senescent cells accumulate with age in mitotic tissues (Dimri et al.), it is not likely that they would entirely populate any tissue, even in very old tissues. It was thus important to ask whether senescent fibroblasts stimulate epithelial cell growth in the presence of presenescent fibroblasts. If this were the case, it would also be essential to evaluate how many senescent fibroblasts are necessary to stimulate the proliferation of epithelial cells. With this in mind, presenescent and senescent fibroblasts were mixed at different ratios, plated, left to attach overnight, and washed with serum-free medium. SCp2 breast epithelial cells were plated in direct co-culture with the fibroblasts and their proliferation was evaluated after 5 to 8 days. A stimulatory effect of senescent fibroblasts on SCp2 colony size was detectable even in the presence of only 10% senescent fibroblasts, resulting in a 50-100% increase in cell number. This effect rose proportionally with the fraction of senescent fibroblasts in the culture, with a maximum of 3 to 6 fold increase in cell number occurring in the presence of 100% senescent fibroblasts. One very important implication of this finding is that the accumulation of even a few senescent cells in an aging organism may stimulate unconstrained epithelial growth and thus contribute to malignant transformation.

Fibroblasts induced to senesce by p14^{ARF} expression stimulate epithelial cell proliferation. We recently showed that expression of the tumor suppressor gene p14^{ARF} induces a senescent-like phenotype in human fibroblasts (Dimri et al., *Molec. Cell. Biol.*, 20(1):273-85). To test the hypothesis that p14^{ARF}-induced senescent-like fibroblasts will effect epithelial cell growth similarly to replicatively senescent fibroblasts, human skin 82.6 fibroblasts were infected with a retrovirus expressing human p14^{ARF} (pBABE-ARF) and used for direct co-culture experiments with HaCAT immortal human keratinocytes. Telomerized 82.6 skin fibroblasts were used in control co-cultures. Our results indicate that human fibroblasts induced to senesce by p14^{ARF}

stimulate epithelial cell proliferation in a manner similar to that observed with replicatively senescent fibroblasts. Interestingly, it has recently been reported (Serrano, M. et al., *Cell*, 88(5):593-602) that expression of oncogenic ras in normal human fibroblasts also induces a senescence-like growth arrest. We are currently investigating whether oncogenic ras-expressing fibroblasts have a similar effect on epithelial cell growth as replicatively senescent fibroblasts. Our preliminary results obtained using WI38 fibroblasts expressing oncogenic ras indicate that indeed this may be the case.

An immediate implication of this is that induction of senescence, usually regarded as a tumor suppressive mechanism that guards cells from malignant transformation, may also contribute to carcinogenesis of surrounding cells through induced changes in microenvironment. It is highly likely that some of the pro-carcinogenic characteristics of the senescent fibroblasts may be present independent of the way senescence was achieved. On a broader scale, this supports our hypothesis that senescence is an antagonistically pleiotropic trait: protecting the organism from cancer early in life, but promoting cancer progression in aged organisms.

Both soluble and insoluble secreted factors contribute to senescent fibroblasts' effect on epithelial cell proliferation. Senescent and presenescent fibroblasts can influence epithelial cells in multiple ways: by cell-cell interactions, through secretion of soluble factors or insoluble extracellular matrix. The effect of the soluble factors secreted by fibroblasts was studied in a two-chamber co-culture system, which does not allow direct contact between epithelial and fibroblast cells, but permits free flow of secreted soluble factors through a porous membrane (0.4μm pore size). Senescent fibroblasts secrete soluble factors that stimulate HaCAT and SCp2 cell growth, even under conditions that prevented direct contact. However, the proliferative effect became significant only between days 6 and 7 of two-chamber co-culture, while this occurs around day 5 of direct co-culture. In addition, while there was about a 3-fold stimulation of colony growth in the presence of senescent relative to presenescent soluble factors (similar to the 3.5-fold stimulation observed in the direct co-culture from the same experiment), the total effect on growth was about 12-fold lower than that observed when direct contact between the two cell populations was allowed.

In order to determine influence of fibroblasts' insoluble matrix on epithelial cell growth, presenescent and senescent WI38 fibroblasts were plated and left to deposit matrix for 2-3 days under serum-free conditions. Fibroblasts were removed by EDTA or NP40 treatment to avoid protein degradation by trypsin. Immunocytochemical analysis of the remaining matrix revealed intact fibronectin deposits, suggesting that the extracellular matrix was intact. SCp2 cells were subsequently plated on the matrix. While SCp2 exhibit 2-5 fold greater proliferation on matrices produced by senescent, relative to presenescent fibroblasts, this represents about 40% of the total growth effect observed with the direct co-culture. This indicates that senescent fibroblasts secrete insoluble components that significantly contribute to the observed stimulation of epithelial cell proliferation. However, the effects observed with direct co-culture were larger than the sum of stimulation observed with the matrix and soluble components separately. This may be due to the ability of epithelial cells in direct co-culture to experience local high concentration of active substances in the close proximity of fibroblasts that produce them. It is also possible that preparation of the matrix denatured some of the active components, making them ineffective. In addition, we can not exclude the potential contribution of direct cell-cell contact, which can only occur in direct co-culture system.

Tumorigenesis assays in nude mice. Since we have established that in a co-culture system senescent fibroblasts stimulate epithelial cell proliferation, we extended our studies using *in vivo* tumorigenicity tests in nude mice. These experiments were not presented in our original Statement of Work, but were mentioned as a possible next phase if the proposed *in vitro* experiments were accomplished and supported our hypothesis. We did not anticipate that we will reach this phase before the end of the fellowship. However, since *in vitro* experiments were advancing faster than predicted and showed very strong effects, we decided to proceed with this next phase immediately and test whether the *in vivo* results will support our culture data. All the procedures were done according to an approved animal protocol.

Presenescent or senescent fibroblasts were mixed with HaCAT keratinocytes and injected subcutaneously into nude mice. Tumor size was monitored for up to 50 days. As a control, HaCAT cells alone were injected into nude mice and no tumors

were observed for up to six months post-injection (0/8, two separate experiments), as reported in the literature (Skobe, M and Fusenig, N.E., *PNAS*, 95(3):1050-5). The presence of fibroblasts, whether presenescent or senescent, stimulated HaCAT cells to form tumors. However, there was a significant difference ($P<0.05$) in the maximal tumor size reached by HaCAT tumors formed in the presence of senescent fibroblasts. The data were compiled from two separate experiments with a total of 15 animals per group. Median size of the tumors formed was two fold larger in the senescent, compared to presenescent, fibroblasts tumor group. In addition to the effect on the maximal size reached by tumors, senescent fibroblasts also stimulate the formation of the tumors. Tumors in the senescent group appear more frequently and earlier than in the presence of presenescent fibroblasts. Moreover, even fibroblasts induced to senesce through expression of $p14^{ARF}$ induced tumorigenesis, albeit to a lower degree than replicatively senescent fibroblasts. The lower tumorigenic potential may be due to the presence of a subpopulation of $p14^{ARF}$ expressing fibroblasts that have not reached high enough expression to achieve a senescent phenotype. Indeed, the labeling index of ARF fibroblast population dropped only down to 33%, implying that one third of the population was still dividing. In replicatively senescent cells, the labeling index reached 15% a week after injections and was below 10% in subsequent weeks. Tumorigenesis experiments using breast epithelial cell co-injections with presenescent and senescent fibroblasts into mouse breasts are in progress.

These data indicate that both replicatively and $p14^{ARF}$ senescent fibroblasts can significantly contribute to breast tumorigenesis *in vivo*. Taken together with our co-culture results, this confirms our hypothesis that senescence can, independent of the mechanisms by which it was achieved, create a tumorigenic microenvironment and thus contribute to the increase in tumorigenesis and malignant transformation in aging individuals.

Key Research Accomplishments

1. Senescent human fibroblasts stimulate 2-6 fold breast epithelial cell proliferation relative to presenescent fibroblasts.
2. Senescent fibroblast-derived extracellular matrix contributes about 40% to the increase in breast epithelial cell proliferation.
3. There is about 3 fold stimulation of colony growth in the presence of senescent relative to presenescent soluble factors. Total effect on growth is about 12 fold lower than that observed when direct contact between two cell populations is allowed.
4. The effect of senescent fibroblasts on breast epithelial cell proliferation is observable even in a mixed pre- and senescent fibroblast population. There is 50-100% increase in growth with only 10% senescent fibroblasts present.
5. Fibroblasts induced to senesce by $p14^{ARF}$, and possibly oncogenic ras, expression stimulate epithelial cell proliferation.
6. Senescent fibroblasts stimulate tumorigenesis. In addition to increase in tumor frequency, there is a significant difference ($P<0.05$) in maximal tumor size reached by epithelial tumors formed in the presence of presenescent and senescent fibroblasts.

Reportable Outcomes

- Poster presentation and Young Investigator Award at American Association for Cancer Research Annual Meeting. April 2000. San Francisco, CA. Krtolica, A., D. Yip, G. Dimri, P. Y. Desprez, and J. Campisi. *"The Double-edged Sword of Replicative Senescence: Senescent Fibroblasts Stimulate Pre-malignant Epithelial Cell Growth."*
- Judith Campisi: "Cellular Senescence and Tissue Microenvironment" presented at Extracellular Matrix and Aging Workshop sponsored by National Institute on Aging, September 1999.
- Manuscript in preparation.

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